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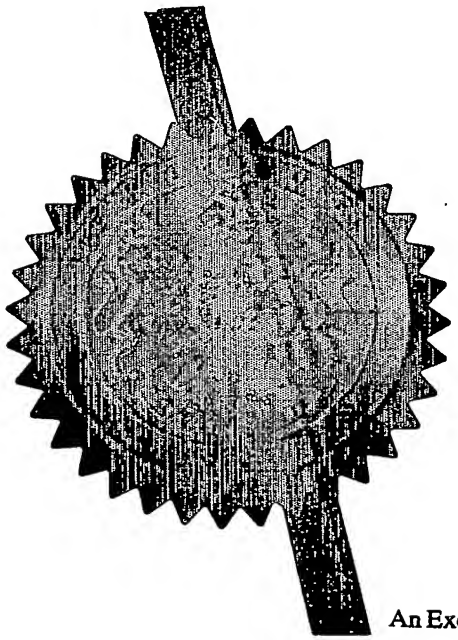

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1. Your reference — PA4490

2. Patent application number *(The Patent Office will fill in this part)* — 23 MAY 2002 — 0211849.5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

MOK, Kenneth Hun
1 Varsity Place
John Towle Close
OXFORD
OX1 4TY

Patents ADP number *(if you know it)* — 8032997001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — IMPROVEMENTS TO RETRO-INVERSO ISOMERS

5. Name of your agent *(if you have one)* — **SOMMERVILLE & RUSHTON**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Description	9
Claim(s)	2 DML
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IMPROVEMENTS TO RETRO-INVERSO ISOMERS

TECHNICAL FIELD

The present invention involves therapeutic peptides that possess inhibitory activity towards elevation of serum triglyceride (triacylglycerol) levels, an unavoidable result that occurs upon ingestion of meals containing high composition of fat. Administration of such peptides before or concomitantly with meals allows for less net absorption of fatty acids into the system, thereby contributing to the prevention of various known cardiovascular diseases as well as obesity-related ailments in general.

BACKGROUND ART

High serum triglyceride level, independent of the well-known risk factor of serum cholesterol, has been regarded as an additional risk factor for developing cardiovascular diseases, including coronary heart disease (Austin MA, Am J Epidemiol 129: 249-59,1989) and atherosclerosis (Patsch JR et al, Arterioscler Thromb 12: 1336-45, 1992). A number of pharmaceutical developments have been made to restrict the elevation of serum triglyceride levels to prevent such cardiovascular ailments.

More significantly, the excessive intake of lipid with respect to energy expenditure leads to obesity, which is currently being regarded as one of the prime health concerns in the Western World. Obesity is a complex medical disorder with implications for diabetes, high cholesterol, cardiovascular conditions, some forms of cancer, and is a major cause of premature mortality. Dietary restriction and behavioural changes is key to prevent obesity, however it is now becoming evident

that the success in preventing or treating obesity can be increased with pharmacotherapy. Several drugs have been developed to combat obesity, however most of these are central-nervous system (CNS)-active, and hence have high abuse potential. Therefore, it would be desirable to have a pharmaceutical agent that would not have these dependency complications.

Recently, a group of low molecular weight peptides which were originally obtained and purified from a non-specific enzymatic proteolysate preparation of bovine reticulocyte protein has been shown to inhibit the elevation of serum triglyceride levels (US Patent 5,958,885). The peptides isolated are low molecular weight, i.e. 3-4 residues in length, and are comprised solely of natural amino acids.

Now, retro-inverso technology, in which oligopeptides are synthesised that are similar to naturally occurring oligo-peptides but with mirror image amino acids put in reverse sequence order (Chorev M, Goodman M, TIBTECH 13:438-45, 1995), is a technology that has had limited take-up in recent years. By utilising non-natural D-amino acids instead of L-amino acids, it can provide an advantage in bioavailability due to inherent resistance against various natural proteases *in vivo* but there is no expectation for its use to effectively mimic or better the biological action of naturally occurring peptides.

SUMMARY OF THE INVENTION

According to a first aspect of the present invention there is provided a pharmaceutical composition for administration to a human or an animal comprising , as an active component, a peptide consisting of: D-Pro D-Tyr D-Val D-Val; D-Pro D-Tyr D-Val; or D-Leu D-Thr D-Val.

These peptides have unexpectedly been found to have a biological activity to reduce serum triglycerides that in, the cases of D-Pro D-Tyr D-Val and D-Leu D-Thr D-Val, respectively, exceed or substantially match the activity of the corresponding natural oligo-peptide. In the case of D-Pro D-Tyr D-Val D-Val the activity is effectively double that of the natural oligo-peptide.

Unlike the natural oligopeptides, the peptides obtained from the retro-inverso chemical synthesis process are also available for functional group modifications if required. This modification further allows for greater specificity and selectivity, and will also permit to tailor the activities of the peptides to suit the patient based on his/her intake of fat composition.

The peptide may, within the scope of the claimed invention, have minor modifications of a nature that is compatible with biological systems, suitably including phosphorylation, sulphonation or iodination of the D-Tyr and / or D-Thr . The D-Pro may , for example, be hydroxylated. Widely used automatic solid-phase peptide synthesis methods of performing this modification include N-alpha-acetylation or N-alpha-formylation for eliminating the positive charge of the N-terminus. When desiring to eliminate the negative charge on the C-terminus, a C-terminal carboxamide or alcohol ester can be readily generated by adopting standard solid-phase peptide synthesis resins.

For optimal activity, the peptide may be modified at the N or C, and suitably both, terminals of the peptide.

Complete reversal of ionisation state can also be straightforwardly performed. The N-terminal NH_2 group is suitably replaced with a COOH group and the C-

terminal COOH group is suitably replaced with an NH_2 group. This modification may suitably be undertaken using one of the conventional techniques for this purpose.

A route for this modification involves:

(1) a C-2 substituted malonyl (or malonamyl) residue substitution for the N-terminal retro-inverso peptide residue, and.

(2) a *gem*-diamino alkyl residue substitution for the C-terminal retro-inverso peptide residue.

The pharmaceutical composition is suitably provided in a form selected from the group consisting of tablets, powder, granules, pills and injectable form. If provided in injectable form it is suitably selected from the group consisting of solutions, suspensions and emulsions.

The said injectable form may be administered by intravenous, intramuscular, subcutaneous, intracutaneous and intraperitoneal administration. The pharmaceutical composition suitably comprises from about 1 to about 100 mg of said pharmaceutical composition.

According to a second aspect of the invention there is suitably provided a food composition for administration to a human or an animal comprising a peptide consisting of D-Pro D-Tyr D-Val D-Val or D-Pro D-Tyr D-Val or D-Leu D-Thr D-Val as an active component.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

Chemical Synthesis and Purification of Peptides

Peptides of the amino acid sequences shown in the sequence listing were synthesised on an Applied Biosystems/Perkin-Elmer 432A Synergy Peptide Synthesizer using FastMoc cycles. The synthesis chemistry involves 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/ piperidine activation, and uses dimethylformamide (DMF) / N-methylpyrrolidine (NMP)/dimethylsulfoxide (DMSO) as the coupling solvent. Synergy Fmoc-Amide resin (Applied Biosystems/Perkin-Elmer) or Rink amide methylbenzhydrylamine (MBHA) resin (Novabiochem) was used for the solid-phase support. The constituting N-α-9-fluorenylmethoxycarbonyl (Fmoc) protected D-amino acids (N-α-Fmoc-D-proline, N-α-Fmoc-O-t-butyl-D-tyrosine, N-α-Fmoc-D-valine, N-α-Fmoc-D-leucine, N-α-Fmoc-O-t-butyl-D-threonine) were from Novabiochem. The peptides were cleaved by adding 1.8 ml of trifluoroacetic acid (TFA) with 0.1 ml of 1,2-ethanedithiol (EDT) and 0.1 ml of thioanisole as scavengers for 1 hour, then precipitated with 15 ml of methyl tert-butyl ether (MTBE) at 4°C and centrifugation at 2000 xg. The MTBE washing was repeated three more times, and the peptides were solubilized with 20% acetic acid. To use as reference compounds, L-Val-L-Val-L-Tyr-L-Pro, L-Val-L-Tyr-L-Pro, and L-Val-L-Thr-L-Leu peptides were prepared using the same methodology.

When necessary, purification of the peptides was performed using preparative reversed-phase HPLC. A Kromasil (Registered Trade Mark) KR-100-10-C8 (10 mm x 250 mm, C8, 10 µm, 100 A, Akzo Nobel) Column was used, with a linear gradient

of 5 to 20% acetonitrile (CH_3CN) in 0.1% TFA over 20 column volumes. The fractionated peak was checked for purity using a Vydac (Registered Trade Mark) 218TP52 RP-HPLC column (2.1 mm x 250 mm, C8, 5 µm, 300 A) with a linear gradient of 1 to 25% CH_3CN in 0.1% TFA. The final purity of each peptide was greater than 97%. MALDI-TOF mass spectrometry analyses using cinnapinic acid as matrix on a Kompact Research MALDI IV instrument (Kratos Analytical) confirmed the identities of the peptides.

Example 2

Oral Administration of Chemically Synthesised Peptides to Determine Inhibition of Triglyceride Level Elevation After Feeding

Olive oil (10 g/kg body weight) was mixed in an emulsion with one type of peptide for each experiment, and administered orally to male ICR mice (6-week old, body weight: 25-28 g) which were fasted overnight. Blood was collected from vena cava under pentobarbital anaesthesia (60 mg/kg body weight, intraperitoneal administration) and serum triglyceride levels were determined using the Triglyceride G Test Kit (Wako Pure Chemical Industries Ltd., Japan). A dose-response curve was obtained, and the 50% inhibition dose ID_{50} were calculated. The results were compared with L-Val-L-Val-L-Tyr-L-Pro (Reference Peptide 1), L-Val-L-Tyr-L-Pro (Reference Peptide 2), and L-Val-L-Thr-L-Leu (Reference Peptide 3) and shown in Table 1.

Table 1

Peptide	ID_{50} (mg / mouse)
SEQ ID.No 1 (D-Pro D-Tyr D-Val D-Val)	0.016
Ref. Peptide 1(L-Val-L-Val-L-Tyr-L-Pro)	0.059
SEQ ID No 2 (D-Pro D-Tyr D-Val)	0.29
Ref. Peptide 2 (L-Val-L-Tyr-L-Pro)	0.39
SEQ ID No 3 (D-Leu D-Thr D-Val)	0.48
Ref. Peptide 3 (L-Val-L-Thr-L-Leu)	0.41

As shown in Table 1, SEQ ID NO 1 and SEQ ID NO 2 display lower ID_{50} values and hence higher specific activities in lowering elevated triglyceride levels than Reference 1 and 2, respectively. SEQ ID NO 3, although slightly less active than Reference Peptide 3, nevertheless exhibits demonstrably similar serum triglyceride lowering activity. These findings are further reinforced by the follow-up experimental results that are summarised in Table 2 below, in which the same names are used for the peptides as above.

As noted previously, for enhanced efficacy the retro-inverso peptides suitably have modified N and C terminals, where the N-terminal of the retro-inverso peptide is converted to replace the NH_2 group with a COOH group and the C-terminal COOH group is replaced with an NH_2 group. This modification may be achieved by carrying out a C-2 substituted malonyl (or malonamyl) residue substitution for the N-terminal retro-inverso peptide residue (Residue R1 in Fig.1), and a *gem*-diamino

alkyl residue substitution for the C-terminal retro-inverso peptide residue (Residue R4).

Figure 1 shows the structural differences between the retro-inverso peptide H-$_D$-R1-$_D$-R2-$_D$-R3-$_D$-R4-OH (Structure I) and the end-group modified peptide HO-*m*R1-$_D$-R2-$_D$-R3-*g*R4-H (Structure II, where *m* and *g* denote malonyl and *gem*-diaminoalkyl residues, respectively).

Procedures to accomplish these end group modifications are well-documented, and are reviewed in Fletcher MD, Campbell MM, Chem Rev 1998, 98: 763-795 and Chorev M, Goodman M, Acc Chem Res 1993, 26:266-73.

According to the present invention, it becomes possible to prevent hyperlipemia in human and domestic animals upon administration of the peptides found in the sequence listing. Such treatment is now known to have far reaching benefits, including but not restricted to, cardiovascular ailments such as hypertension and arteriosclerosis, and obesity-related complications in general.

Table 2

	Peptide Dosage (mg / mouse)	Serum Triglyceride (mg / 100 ml)					
Distilled Water Only	-	47					
Olive Oil Only	-	511					
		SEQ ID No 1	Ref. Peptide 1	SEQ ID No 2	Ref. Peptide 2	SEQ ID No 3	Ref. Peptide 3
Olive Oil + Peptide	0.005	490	506	510	521	488	522
	0.01	274	395	490	458	477	487
	0.02	218	386	456	472	486	456
	0.05	143	291	411	444	464	457
	0.1	112	199	365	381	372	404
	0.2	57	113	296	353	347	312
	0.4	64	28	201	239	262	222
	0.6	60	63	145	215	239	209
	0.8	55	50	62	161	203	163

CLAIMS

1. A pharmaceutical composition for administration to a human or an animal comprising a peptide consisting of D-Pro D-Tyr D-Val D-Val or D-Pro D-Tyr D-Val or D-Leu D-Thr D-Val as an active component.

2. The pharmaceutical composition of claim 1 being selected from the group consisting of tablets, powder, granules, pills and injectable form.

3. The pharmaceutical composition of claim 2 which is an injectable form.

4. The pharmaceutical composition of claim 3 wherein said injectable form is selected from the group consisting of solutions, suspensions and emulsions.

5. The pharmaceutical composition of claim 3 wherein said injectable form may be administered by intravenous, intramuscular, subcutaneous, intracutaneous and intraperitoneal administration.

6. The pharmaceutical composition of claim 1 wherein the composition comprises from about 1 to about 100 mg of said peptide.

7. A food composition for administration to a human or an animal comprising a peptide consisting of D-Pro D-Tyr D-Val D-Val or D-Pro D-Tyr D-Val or D-Leu D-Thr D-Val as an active component.

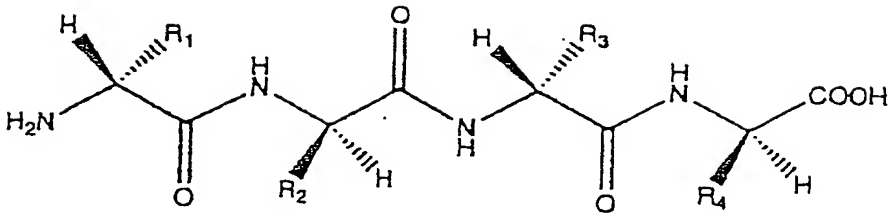
8. A pharmaceutical composition as claimed in any of claims 1 to 6, wherein the N-terminal NH_2 group is replaced with a COOH group and / or the C-terminal COOH group is replaced with an NH_2 group.

5

ABSTRACT

RETRO-INVERSO ISOMERS

In a first aspect the present invention provides a pharmaceutical composition for administration to a human or an animal comprising a peptide consisting of D-Pro D-Tyr D-Val D-Val or D-Pro D-Tyr D-Val or D-Leu D-Thr D-Val as an active component. This has been found to give great efficacy in lowering serum triglyceride levels.



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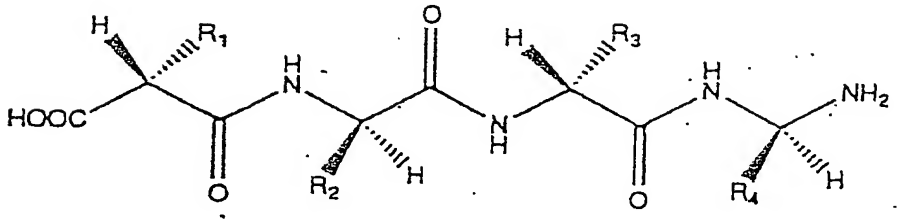

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Figure 1.